KARPAGAM ACADEMY OF HIGHER EDUCATION

DEPARTMENT OF BIOTECHNOLOGY

III B.Sc., BIOTECHNOLOGY – SEMESTER 6

LECTURE PLAN –Bioanalytical Tool Practical (**16BTU611B)**

S.No	Lecture Duration (hr)	Experiments to be covered	Support materials
1	1	Native gel electrophoresis of proteins	T2 Pg 119
2	1	SDS-polyacrylamide slab gel electrophoresis of proteins under reducing conditions.	T1 Pg 79-84
3	1	Preparation of the sub-cellular fractions of rat liver cells	T2 Pg 394
4	1	Preparation of protoplasts from leaves	T2 Pg 512
5	1	Separation of amino acids by paper chromatography.	T3 Pg 261
6	1	To identify lipids in a given sample by TLC.	T3 Pg 264
7	1	To verify the validity of Beer's law and determine the molar extinction coefficient of NADH.	T3 Pg 349-352

References

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SYLLABUS

1. Native gel electrophoresis of proteins
2. SDS-polyacrylamide slab gel electrophoresis of proteins under reducing conditions
3. Preparation of the sub-cellular fractions of rat liver cells
4. Preparation of protoplasts from leaves
5. Separation of amino acids by paper chromatography.
6. To identify lipids in a given sample by TLC
7. To verify the validity of Beer's law and determine the molar extinction coefficient of NADH

1. Native gel electrophoresis of proteins

1. Aim

To perform agarose gel electrophoresis of isolated DNA.

2. Introduction

Electrophoresis is the process of separating ionically charged molecules based on their charge and/or size. This method utilizes Agarose, a purified linear galactan hydrocolloid isolated from agar or agar bearing marine algae, for gel preparation. When agarose is heated with water or standard buffer to boiling point and then allowed to cool, it forms a gel by hydrogen bonding. During the process of gelling, pores are formed and the pore size depends upon the concentration of agarose. Low concentration of agarose results in large pores allowing high molecular weight DNA to move through. Conversely, high concentration results in smaller pores which are suitable for low molecular weight DNA.The principle behind this experiment is that the rate of electrophoretic movement of DNA depends upon its size. In general, bigger DNA molecules move slowly compared to small size DNA molecules. Circular plasmid move slowly than linear plasmid. When electric charge is applied, DNA being negatively charged, moves towards the anode, while passing through the pores of the gel (Fig. 1). The larger DNA molecules move slowly through the gel, while smaller ones moves faster and hence gets separated faster into bands of different size at different positions on the gel, which can be further evaluated.

3. Material Required:

Power supply

Smoke wells

Buffer solution

Electrophoresis tank

Θ electrode

Θ electrod

Usually, TAE/TBE buffer in Mili-Q water is used to prepare the gel as DNase and RNase in normal water can denature the nucleotide molecules. A loading dye is mixed with the DNA samples. It generally contains a dye to assess how "fast" the gel is running and a reagent to render the samples denser than the running buffer (so that the samples sink in the well).When a DNA–ethidium bromide complex is illuminated with UV-β rays (280-320 nm), part of it is absorbed and rest is irradiated back in the range of 590-620 nm, as a result of which DNA-ethidium bromide complex appears as orange colour band.

3. Materials Required

3.1 Biological Material: DNA sample.

3.2 Chemicals/Reagents: TAE buffer -(Tris, EDTA, glacial acetic acid, NaOH:-stock solution – 50X, working solution – 1X), agarose, bromophenolblue dye/ 6X gel loading dye, ethidium bromide, 100 kb/1 kb DNA size marker and distilled water.

3.3 Equipment: Submarine gel electrophoretic system with power supply unit, UV transilluminator with face shield.

3.4 Glassware/Plasticware: Micropipettes (Accupipette T-20 or Gilson-P-20), glass tray, tissue paper, parafilm, cello tape etc.

4. Preparation of Reagents

i. 50X TAE (stock solution): Prepare 50 X TAE solutions by dissolving 242 gm Tris, 100 mL 0.5 M EDTA (pH 8.0) and 57.1 mL Glacial Acetic acid, and adjust the volume to 1000 mL by adding double distilled water. As EDTA does not dissolve easily, it is to be put in a magnetic stirrer for about 5 hours (NaOH can be added to balance pH to accelerate dissolving). The buffer thus prepared is to be autoclaved.

ii. 1X TAE (working solution): A working solution is prepared by 1:49 dilution (20 mL stock solution of 50X TAE + 980 mL double distilled water).

iii. Agarose Gel: 0.8% Agarose gel is prepared by dissolving 800 mg Agarose in 100 mL 1X TAE buffer. Boil the solution to dissolve Agarose. When the solution cools down a bit, 2 µL ethidium bromide is added to it and casted in an electrophoretic casting plate and an electrophoretic comb

is placed at an end of the gel in a way that the legs of the comb remain inside the liquefied gel. It is then allowed to solidify.

iv. DNA sample for loading: 12 µL DNA sample is mixed with 2 µL (approx.) 6X Gel Loading dye and loaded in

the gel.

5. Procedure

Placing the gel and loading the sample:

i. Remove the comb slowly, after the gel gets solidified, leaving behind fine wells in the gel. Place the solidified agarose gel, along with the casting tray, place inside the electrophoresis chamber keeping the wells towards the cathode.

ii. Fill the electrophoretic tank with 1X TAE buffer to such an extent that the gel remains submerged.

iii. Load the DNA samples along with molecular size markers into the wells with the help of T-20 micropipette at

the cathode end.

iv. Run the gel till the time the tracking dye covers more than ¾ distance in the gel.

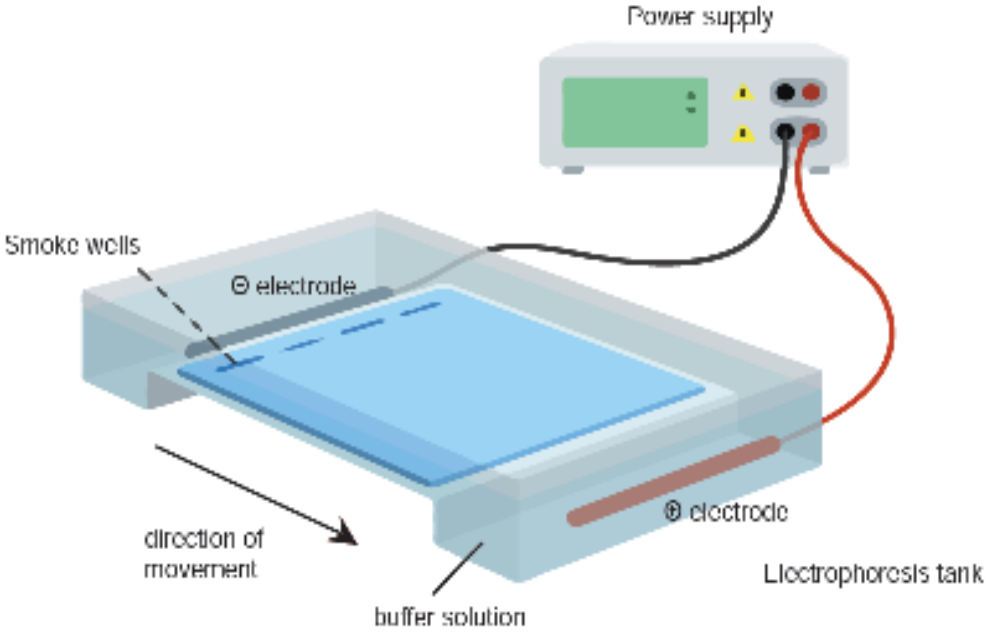

Fig. 1: Illustration of DNA electrophoresis equipment used to separate DNA fragments.

6. Precautions

i. Generally 0.8% agarose gel is used.

ii. For low molecular weight DNA (i.e. ≤ 1.5 kb, like amplified product of PCR) gel strength should be 1.3-1.5%.

iii. Care should be taken not to switch the leads on the power source, as it will result in the sample running backwards.

iv. Gel should be carefully transferred from the chamber/tray on to the transilluminator to avoid dropping the gel or breaking in between.

v. Ethidium bromide is a strong carcinogen. Chemically treat it as per standard protocol for bio hazardous chemicals before disposal.

vi. Wear hand gloves during the entire course of operation.

vii. Care should be taken not to run the gel for too long as it can exhaust the buffering capacity of the solution.

Result: The Protein is electrophoresised through Native gel electrophoresis.

2. SDS-polyacrylamide slab gel electrophoresis of proteins under reducing conditions

1. Aim

To analyse proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2. Introduction

Polyacrylamide gel electrophoresis (PAGE) is widely used technique to separate biological macromolecules, usually proteins or nucleic acids, on the basis of their electrophoretic mobility i.e. a function of the length, conformation and charge of the molecule. Molecules may be run in their native state, preserving the molecules higher-order structure, or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. Sodium dodecyl sulphate (SDS) an anionic detergent is used to denature proteins and to impart a negative charge to the linearized proteins. This procedure is called SDS-PAGE. When a protein mixture is heated to 100 ℃ in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density that is same net negative charge per unit weight. The electrophoretic mobilities of these proteins are a linear function of the logarithms of their molecular weights. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure.

This is known as native PAGE. Following reagents are added for preparation of gel:-

a) Acrylamide, when dissolved in water, slow, spontaneous auto polymerization takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating system greatly accelerates polymerization.

b) Bisacrylamide (N,N′-Methylenebisacrylamide) is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to

one another, thereby resulting in a gel. SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides.

c) Ammonium per sulfate (APS) is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.

d) TEMED (N, N, N′, N′-tetramethylethylenediamine) stabilizes free radicals and improves polymerization. The rate of polymerization and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals result in decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerisation in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10 mM.

3. Materials Required

3.1. Chemicals/Reagents: Acrylamide/Bisacrylamide, 10% (w/w) SDS, 1.5 M Tris-HCl (pH-8.8), 0.5 M Tris-HCl (pH- 6.8), sample buffer (5X), 5X electrode (running) buffer, 10% APS, TEMED, staining solution, de-staining solution.

3.2. Equipment: Electrophoretic unit, power bank, water bath, rocker.

3.3. Glassware/Plastic ware: Micropipette, microtips, microcentrifuge tubes.

4. Reagents Preparation

i. Acrylamide/Bis (30% T, 2.67% C):

Acrylamide (29.2g/100 mL)

N'N'-bis-methylene-acrylamide (0.8 g / 100 mL)

Make 100 mL with deionized water. Filter and store at 4^0C in the dark.

ii. Sample Buffer (5X):

0.5 M Tris-HCl, pH 6.8-1.0 mL

Glycerol- 0.8 mL

10% (w/v) SDS- 1.6 mL

0.5%(w/v) bromophenol blue - 0.2 mL

β-Mercaptoethanol- 0.4 mL

Deionized Water- 4.0 mL

iii. 5X Electrode (Running) Buffer:

Tris base- 1.5 g

Glycine- 7.2 g

SDS- 0.5 g

Deionized Water- 100 mL

iv. Staining Solution:

0.1% (w/v) Coomassie Brilliant Blue

20% (v/v) Methanol

10% (v/v) Acetic acid

v. De-staining solution:

5 parts of methanol

1 part of acetic acid

4 parts of water

5. Procedure

Assemble the SDS-PAGE apparatus

Components	For 10% resolving gel Volume	For 4% stacking gel Volume
30% Monomer	3.3 mL	1.3 mL
Tris-Cl Buffer (pH 8.8)	2.5 mL	-
Tris-Cl Buffer (pH 6.8)	-	2.5 mL
Water	4.04 mL	6.04 mL
10% SDS	100 µL	100 µL
10% APS	50 µL	50 µL
TEMED	10 µL	10 µL
Total		10 mL

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i. Prepare 10% resolving gel and 4% stacking gel were prepared as per the table given below

ii. Pour the resolving gel mixture in between the glass plates of the apparatus, leaving a space of 1cm for the stacking gel. Fill this space with water to overlay the gel and obtain a uniform surface. Leave the gel to polymerize for 45 minutes.

iii. After the polymerization of the resolving gel, pour off the overlaying layer of water, and over the resolving gel pour the stacking gel. Insert a comb of 0.75 mm thickness into the stacking gel, taking care to avoid trapping air bubbles. Leave the stacking gel undisturbed for 40 minutes to polymerize.

iv. Mix the sample and the gel loading buffer in the ratio of 4:1 as the gel loading buffer is of 5X concentration and the required concentration is 1X. Mix 5 μL of the gel with 20 μL of sample in a micro centrifuge tube and boil at 90-100 _C to allow SDS to bind to proteins for 5 minutes.

v. Perform the electrophoresis at 150 V for 45 minutes to1hour.

vi. After electrophoresis, immerse the gel in the staining solution for 30-40 minutes, followed by de-staining overnight.

vii. The gel was then observed for the bands developed for the proteins in the sample mixture.

6. Observation and Result

The proteins will be observed on polyacrylamide gel as distinct blue bands.

7. Precautions

i. Do not allow the gel to dry after polymeraization

ii. Take out the comb carefully, making sure the well are not disturbed.

iii. Keep a track of the gel while running.

iv. Do not inhale SDS, as it is a neurotoxin and harmful when ingested and irritating for eyes

v. β-Mercaptoethanol is a harmful and poisonous material. Avoid contact with eyes

vi. Acrylamide is a potent cumulative neurotoxin: wear gloves at all times

3.Preparation of the sub-cellular fractions of rat liver cells

1. Introduction

All eukaryotic cells contain organelles that perform specific functions. **Cellular fractionation** is a technique that enables the researcher to separate and isolate bulk quantities of organelles and other cellular components. Once isolated, the functioning of the cellular constituents can be studied more easily than within the intact cell.

Fractionation begins with **homogenization** which involves the disruption of the cells by mechanical shearing, ultrasound, osmotic shock, or detergents such as SDS. The process releases the cellular organelles which can then be separated from each other by **centrifugation**. Centrifugation at low speeds causes the sedimentation of only the largest and densest of components. These components can be collected and the unpelleted portion, or supernatant, can be centrifuged again at higher speeds and for longer durations to pellet successively smaller and smaller components. Once isolated, the components can be assessed for purity by light or electron microscopy and their biochemical activities assayed to determine structure/function relationships.

In the following exercise, you will fractionate rat liver cells to isolate cellular fractions enriched in nuclei, mitochondria, and cytoplasm. An overview of the procedures is given in Figure 4.1

2. Materials Required

rat liver (on ice), sucrose buffer, large Petri dish, scissors, razor blades, micropipettors, homogenizers, beakers, cheese cloth, centrifuge tubes (50 mL), microfuge tubes, polypropylene tubes, methyl green pyronin, slides, coverslips, slide warmer, glycerin, microscope

Procedure

a. Homogenization of rat liver

For the following procedures, both groups from a lab bench will work together as a team (or 6 to 8 students).

1. Samples of fresh rat liver, perfused with ice cold sucrose buffer (0.15 M sucrose, 5 mM $MgCl_2$, 0.01% NP40 detergent, 50 mM NaCl, in 10 mM Tris buffer, pH 7.1), are provided. Pour 20 ml of ice cold sucrose buffer into the large Petri dish provided and add the portion of the rat liver provided by your instructor (approx. 2 g). Chop the tissue into fine pieces using the scissors provided and wash and decant off excess liquid into sink. Rinse the tissue thoroughly twice more using 2 x 20 ml rinses of buffer. After the final rinse, add 15 ml of buffer to the tissue to obtain a tissue: buffer ratio of 1:5 (weight to volume).

2. Pour the suspended tissue into the homogenizer and homogenize on ice until no further lumps of tissue remain (approximately 25 passes of the pestle). Place 10 layers of cheese cloth previously pre-wetted with sucrose (homogenization) buffer over a clean 100 ml beaker. Pour the homogenate through the cheese cloth into the beaker maintained on ice. Rinse the homogenizer with a further 10 ml of ice-cold buffered sucrose and pass the wash through the cheese cloth into the tube. Squeeze the cloth to remove the remaining fluid from the tissue fragments. You should have approximately 20 ml of tissue homogenate with a tissue:buffer ratio of 1:10 (weight to volume). Save aliquots of the crude homogenate as instructed in Table 4.1. **Maintain on ice!** Maintenance on ice prevents degradation and denaturation of the proteins and organelles. The samples will be used for assays and microscopy. Use 10 □L from one of your aliquots to make a slide (see part C).

B. Differential Centrifugation

1. Transfer the chilled sample from the beaker to a 50 ml tube. Balance pairs of tubes to equal weight and centrifuge in a pre-cooled centrifuge (4°C) for 10 min. at 600 x g. Pipette the supernatant (taking care not to resuspend pellet) into a fresh, clean centrifuge tube and maintain on ice.

2. Resuspend the pellet in the centrifuge tube using 20 ml of sucrose buffer by gently agitating with a Pasteur pipette. Recentrifuge at 600 x g for 10 min. Discard the supernatant and

resuspend the pellet in 6 ml of ice-cold sucrose buffer. This fraction is the ***nuclear fraction***. Take 10 ml and prepare a slide as stated in part C. Also, aliquot this sample as described.

3. Centrifuge the supernatant obtained during step 1 for 20 min. at 10,000 x g. Transfer the supernatant into a fresh, ice cold tube. **Estimate the volume and record this value:ml.** This is the ***cytosolic fraction***. Take 10 mL and prepare a slide as stated in part C. Also, aliquot this sample as described.
4. Resuspend the pellet obtained from Step 3 in 10 ml of ice-cold sucrose buffer. This sample is the ***mitochondrial enriched fraction***. Take 10 mL and prepare a slide as stated in part C. Also, aliquot this sample as described.
5. You should have 4 samples: the crude homogenate, nuclear, mitochondrial and cytosolic fractions. Aliquot them as outlined. **Keep these samples on ice.** You will be using these samples in later exercises; see section D for details.

C. Evaluation Of Cellular Fractions

1. Prepare slides stained with methyl green pyronin of all 4 samples using a single drop of material from each tube as described below.

--Place a drop of the fraction on a clean slide.
--Spread the drop with the edge of a second slide to make a smear.
--Allow the slide to air dry with gentle heat on the slide warmer.
--Add 1 drop of methyl green pyronin stain. Let it dry on the warmer for approximately 3 min.
--Immerse slide vertically, in a beaker of DW for 1 min. Do not agitate. Remove and dry carefully on a warmer, making sure the specimen is face up.
--Add 1 drop of glycerin solution and mount the coverslip.

2. Observe in bright field microscopy. Nuclei should stain purple-blue, cytoplasm red or pink, and mitochondria may be seen as small dots. Examine the slide and record your

results in Table 4.2. Use an arbitrary scale (+++/-) to record the degree of cytoplasmic staining.

D. Storage Of Cellular Fractions

Divide the remainder of each of your four samples as shown below in table 4.1. Label the tubes carefully using the permanent markers. Your instructor will store the samples at -80°C. Include your lab section and bench #. These samples will be used in the later exercises.

TABLE 1.

Volumes are to be shared among 1 bench (2 groups)

Cellular Fraction	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Crude homogenate	1.5 mL (protein quant)	1.5 mL (enzy.markers)	Not needed	Not needed	Not needed
Nuclei	1.5 mL (Protein quant)	1.5 mL (enzy.markers)	50 mL (SDS PAGE)	Remainder	Not needed
Mitochondria	1.5 mL (Protein quant)	1.0 mL (enzy.markers)	Remainder (7 mL minimum) (Enzyme Kinetics)	Not needed	Not needed
Cytosolic	1.5 mL	1.5 mL (enzy.markers)	50 mL (SDS PAGE)	200 □L (dif. gene)	Remainder

	(protein quant)				

* Volumes over 1.5 mL require polypropylene tubes. Otherwise, use microfuge tubes!

4. Preparation of protoplasts from leaves

Protoplasts are cells that have had their cell wall removed, usually by digestion with enzymes. Cellulase enzymes digest the cellulose in plant cell walls while pectinase enzymes break down the pectin holding cells together. Once the cell wall has been removed, the resulting protoplast is spherical in shape. Digestion is usually carried out after incubation in an osmoticum (a solution of higher concentration than the cell contents that causes the cells to plasmolyze). This makes the cell walls easier to digest. Debris is filtered and/or centrifugedout of the suspension and the protoplasts are then centrifuged to form a pellet.On resuspension, the protoplasts can be cultured on media that induce cell division and differentiation. A large number of plants can be regenerated from a single experiment—a gram of potato leaf tissue can produce more than a million protoplasts, for example.

PREPARATION OF PROTOPLASTS

Method for isolating large numbers of metabolically competent protoplasts from leaves of monocotyledons (grasses), dicotyledons (such as spinach and sunflower), or from hypocotyl tissue (e.g., *Brassica napus*).

1. Leaf slices of monocots and dicots are prepared by cutting the leaves with a sharp razor blade into segments 0.5–1 mm in size. In the case of dicots, the epidermis can be scraped off before cutting by rubbing with fine

carborundum powder or with a fine nylon brush.

2. Set up 50 mL of digestion medium (for 10–15 g of plant tissue) accordingto the recipe to Solution A.

3. Incubate the leaf slices or pieces in a 19-cm-diameter dish containing thedigestion medium for 3 hours at 25°C, covered with a plastic film. It maybe advantageous to replace the digestion medium at intervals of 1 hour,

as the enzymes might become inactivated by substances released frombroken cells.

4. After completion of the incubation the digestion medium is carefullyremoved and discarded. It normally contains very few protoplasts. The plant tissue is then washed 3 times by shaking gently with 20-mL wash medium (Solution B).

5. After each wash, the tissue is collected by pouring through a tea strainer(0.5- to 1-mm pore size) and the combined washes are then filtered throughnylon mesh (100–200 mm pore size) to remove vascular tissue and
undigested material.

6. The protoplasts are collected by centrifugation of the combined filteredwashes for 3 minutes at 50–100 xg and the supernatant is aspirated anddiscarded.

7. This crude protoplast preparation also contains some cells and chloroplastsand it is important to purify the protoplasts to remove these contaminants.This can be done with solutions of sucrose and sorbitol of different densities.

8. The protoplast pellet is gently resuspended in 40 mL of Solution C, andthis suspension is divided among two 100-mL centrifuge tubes.

9. To each tube, slowly add 5 mL of Solution D and then overlay this with 5 mL of wash medium (Solution B) to make a 3-step gradient.

10. Centrifuge at 300 g for 5 minutes.

11. The protoplasts now collect as a band at the interface between the 2 toplayers. Carefully remove them with a Pasteur pipette.

12. The protoplasts should be examined with a light microscope to ensure that the preparation is free of cells and chloroplasts.

13. When a large portion of the protoplasts is pelleted in this sucrose/sorbitol gradient, the density of the 2 layers can be increased by adding 5%–10% Dextran (15000–20000 Mr) or 10%–20% Ficoll to increase the percentage ofthe floating protoplasts.

14. The purified protoplasts can be concentrated by diluting with 10 mL of Solution B, cenrifuging at 100 g for 3 minutes and then resuspending the pellet in a small amount of medium by gently shaking the tubes.

15. The protoplasts are stable for up to 24 hrs when stored on ice. Photosyntheticactivity of the protoplasts can be determined by measuring codependent evolution with an oxygen electrode, provided rapid stirring is avoided, as this will break some of the protoplasts. A suitable mediumis listed as Solution E.Protoplasts exhibit a relatively broad pH optimum, but at more acidic Ph values the bicarbonate concentration should be lowered.

Solutions

Solution A (Digestion medium)

Composition

For 50 mL use:

500 mM

D-Sorbitol

4.56 g

1 mM

CaCl2

7.35 mg

5 mM

MES-KOH, pH 5.5

49.00 mg

2%

Cellulase Onozuka R10

1.00 g

0.3%

Macerozyme R10

0.15 g

The pH must be adjusted to 5.5 with KOH before adding the enzymes

Solution B (Wash medium)

Composition

For 100 mL use:

500 mM

D-Sorbitol

9.11 g

1 mM

CaCl2

14.70 mg

5 mM

MES-KOH, pH 6.0

98.00 mg

The pH of the solution must be adjusted to 6.0 with KOH.

Solution C

Composition

For 100 mL use:

500 mM

Sucrose

8.56 g

1 mM

CaCl2

7.40 mg

5 mM

MES-KOH, pH 6.0

49.00 mg

The pH of the solution must be 5 mM

adjusted to 6.0 by adding KOH. MES-KOH, pH 6.0

49.00 mg

The pH of the solution must be adjusted to 6.0 by adding KOH.

Solution D

Composition

For 100 mL use:

400 mM

Sucrose

6.80 g

100 mM

D-Sorbitol

0.90 g

1 mM

CaCl2

7.40 mg

Solution E

Composition

For 100 mL use:

500 mM D-Sorbitol 9.10 g

1 MM CaCl2 15.00 mg

30 mM Tricine-KOH 538.00 mg

5 mM NaHCO3 42.0 mg

5.Separation of amino acids by paper chromatography.

1. Aim

To separate amino acids by paper chromatography by ascending method.

2. Introduction

Chromatography is a separation technique that relies on the differential solubility of solutes in solvents. The separation of components from a mixture can therefore be achieved if the solutes are partitioned between two or more phases. Paper chromatography is thus a type of partition chromatography in which the differences in the partition coefficients of the substances to be separated are used for separating them. When solvent system containing both hydrophilic and hydrophobic components (mobile phase) migrates on a paper, the hydrophilic solvent is absorbed on the cellulose, which acts as the stationary phase whereas the hydrophobic one does not. Thus, a phase separation takes place at the micro level. When the solvent front reaches the spot where the compounds have been spotted, the compounds get partitioned as the solvent front migrates further by capillary action and separation is achieved. The separated aminoacids are visualized by spraying a solution of ninhydrin (trihydrindene hydrate) and heating the paper. The amino acid spots appear purple.

The two amino acids proline and hydroxyl proline appear yellow. Ninhydrin deaminates and decarboxylates the aminoacids and at the same time gets reduced to hydrindantin. This hydrindantin then combines with another molecule of ninhydrin and one molecule of ammonia to form a purple coloured complex (Ruhemann's purple reaction). Ratio of distance traveled by the solute to the distance traveled by the solvent from the origin of the spot is termed as

Rf value (resolution front). Different substances have different characteristic Rf values. Protocol Manual **Page 261**

3. Materials Required

3.1. Chemicals/Reagents: Reference amino acids (leucine, lysine and proline), mixture of unknown amino acids,

solvent system, butanol: acetic acid: distilled water in a ratio of 4:1:5, 0.1 % ninhydrin in acetone.

3.2. Equipment: Chromatographic chamber.

3.3. Glassware/Plastic ware: Capillary tubes, hot plate.

3.4. Miscellaneous: Pencil, scale, strip of whatman no.1 chromatography paper (about 15 mm x 200 mm).

4. Procedure

i. Fill the chromatographic chamber with solvent system to height of about 1 cm.

ii. Close the lid and allow equilibrating for at least 30 minutes.

iii. On the end of the chromatographic paper, draw a light horizontal line with pencil about 1.5 cm from the bottom.(This is the base line)

iv. On this line mark 4 equidistant spots. Label these spots as Le (for leucine), Ly (for lysine), P (for proline), and M (for mixture).

v. With the help of capillary tubes, transfer a very small drop of appropriate solution to the spots. Allow to dry.

vi. Insert the chromatographic paper into the chamber in such a way that the end near the spot is immersed in the solvent system and the spots are about 5-6 mm above the solvent level. Close the chamber and allow the solvent to run. Take care not to allow the paper to touch either the bottom or the sides of the chamber.

vii. When the solvent has migrated to about 10-15 cm, remove it from the chamber and mark the solvent.

viii. Dry the paper.

ix. Spray ninhydrin and heat on a hot plate or can be placed in a heated oven.

x. When the spots develop, mark their outlines tightly with pencil.

xi. Measure the distance from the origin (base line) to the solvent front (distance traveled by solvent).

xii. Mark centre of spots, measure the distance traveled by solute and tabulate them.

5. Observations

It is found that:-

i. Leucine with the highest position coefficient migrates the longest distance.

ii. Lysine with the lowest partition coefficient migrates the minimum distance.

iii. Proline occupies a position in between the two on spraying with ninhydrin.

iv. Leucine and lysine develop into dark purple spots with strong heat.

v. Proline which is an amino acid develops yellow colour.

vi. Measure the distance travelled by the solvent system and each amino acid and tabulate.Life Sciences Protocol **6. Calculations**

Calculate the Rf values of each amino acids

Rf = Distance travelled by the solute

Distance travelled by the solvent

7. Results

i. Rf value of Leucine = ----------.

ii. Rf value of Lysine = ----------.

iii. Rf value of Proline = ----------.

8. Conclusion

i. Rf value of given mixture matches with Rf value of reference amino acids such as leucine, lysine and proline.

ii. Leucine is a neutral, hydrophobic amino acid and migrates maximum distance because it has maximum

solubility in butanol and a high partition coefficient.

iii. Proline migrates a relatively closer distance because it has partial positive charge.

iv. Lysine is a hydrophilic amino acid and migrate minimum distance as it is highly soluble in aqueous medium

and least soluble in organic solvent.

8. Precautions

i. Use clean glassware for setting up the experiment.

ii. Do not touch the chromatography paper with hands.

iii. Be very careful while spraying Ninhydrin on the chromatogram (avoid skin contact).

6. To identify lipids in a given sample by TLC

AIM:-Separation of bimolecular by gel prevention chromatography.

REQUIREMENTS:- Chromatographic column, micropipette.

THEORY:- Molecules can be separated on the basis of differences in their size by passing them down a column containing swollen particulars of gel smaller molecule can enter the gel but larger molecular are absorbed from the crossing network. This means that volume of a solvent is very much less for molecules totally excluded from the gel than from the smaller molecular which are free from separation. The separation of the molecules of the column gel preparations passed down the column is that the small molecule diffuse into the column & follow a longer path than larger molecules which are completed excluded from the gel particular complete separation occur with larger molecules clustering first as small molecule in last.

If molecular is completely excluded in gel then kd=0 & if the molecule has complete accessibility to the gel, then kd=1.

Molecule have normal kd values b/w 0 to 1, if kd >1 then absorption of the compound on the gel has occurred column porous bead & sauce is applied on the top of the column of chore beads of the cross linked gel, they can get separated as follows: 1. Large molecules cannot cuter the poses & Clute as 1st peak in the chromatogram. They elute fast & this from total elusion. 2. Intermediate molecules enter the poses & have an average residence time in the particles depending upon their size & shape. This portion of chromatogram is called selective permeation reagent. 3. Surface molecular enter the poses & have longest residence time: elute out as last peak in chromatogram. 4. This last peak is total permeation limit.

PROCEDURE:-

1. Fixed the washed & cleaned column vertically to the stand.

2. Equilibrate the column with gel filtration buffer. Drawn out the buffer completely.

3. Suspended 4g of sephadax (Aluminum) in sodium chloride soln & leave it to swell for 3 hrs. during this time stir the soln & removed any fumes by decantation.

4. Prepare the column of the gel by pouring the gel expansion into a column & allowed to settle under graving while maintain a slow flow rate to column.

5. Equilibrate the column with gel filtration buffer. Drain out the buffer completely.

6. Loaded the known amount of the mixture of methylene blue & cobalt chloride to the top of column & elute it with isotonic solute soln collected the colour fraction in diff tubes. Then fix the bottom cap & top cap to stop the flow of buffer & stored at 4ºC for next use.

PRECAUTIONS:-

1. Do not let the column to gel dry any time.

2. Always open the top cap of the column first & then bottom cap to start the flow of buffer.

3. There should not be any air bubble in the column.

4. Loading should be done carefully

7. To verify the validity of Beer's law and determine the molar extinction coefficient of NADH

AIM:- Demonstrate Beer- Lambert's Law Requirements:- Spectrophotometer, Test tube, Test Tube stand, Breakers, micropipettes, Bromophenol blue and Distilled water.

Principle:- When a ray of monochromatic light with initial intensity 'Io' passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light _I' is small. There is some loss of intensity from scattering by particles in the solution & reflection at the interphase but mainly from absorption by the solution. The relationship between 'I' & 'Io' depends on the path-length of absorbing medium 'l' & the concentration of absorbing medium 'c'.

LAMBERT'S LAW:- When a ray of monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the length of absorbing medium increases.

BEER'S LAW:- When a ray ofWhen a ray of monochromatic light passes through a medium its intensity decreases exponentially as the concentration of absorbing medium increases. These 2 laws are combined together in Lambert-Beer Law.

I=I0e -kcl The ratio of intensity is known as transmittance & is usually expressed as '%' T=I/Io = ekcl Taking log, loge Io/I = KCl log10 Io/I=2.303 KCl log10 Io/I=KCl The expression ―log10 Io/I‖ is known as Extinction (E) or Absorbance (A).

Therefore, E= KCl If the Beer Lambert Law is obeyed & the _l' is kept constant, then plot a graph against _E' & _conc.' will give the straight line passing through the origin whereas a plot of (%) transmittance against conc. gives negative exponential curve.

MOLAR EXTINCTION COEFFICIENT:- If _l' is 1 cm & concentration is 1 mole /L then its absorbance is equal to _K'. Therefore, E=K The molar extinction coefficient which is characteristic of compound has dimensions of 1 mole/cm.

SPECIFIC EXTINCTION COEFFICIENT:- The molar weight of some compounds such as proteins or nucleic acid in a mixture are not readily available & in this case the specific extinction coefficient is used. This is the extinction of 10g/l of the compound in the light part of 1cm.

PROCEDURE:- i. Took 5 test tubes in a stand to make different concentration of dye & mark them as T1, T2, T3, T4, T5. ii. Took Bromophenol Blue solution as a stock. iii. Then made different concentration from the stock solution by adding distilled water. iv. In T1, added 10ml of stock & No water & in T2, 8ml of stock & 2 ml Distilled water to make 10ml, then add 6ml of stock & 4ml Distilled Water in T3 & So On. v. In T6, added Distilled water & no stock, Mark it as blank. vi. Now took absorbance of all the concentrations at particular wave length of 590nm. vii. Noted down the absorbance of different cone & plotted graph b/w absorbance & concentration. Calculations:- Stock solution:- Bromophenol blue = 100 mg/ 100m.

Concentration = Quantity/ volume = 100/100 = 1 mg/ml = 1000µg/ml Working solution:- Volume of stock = 1ml Distilled water = 99ml C1V1 = C2V2 (Stock) (Working solution) 1000x1 = C2x100 C2 = 10µg/ml.

PRECAUTIONS

i.Switch on the spectrophotometer half an hour before use.

ii. There must be no Ionization, association, dissociation, and solvation of the solute with concentration of time.

iii. The solution is the concentration giving intense colour, law holds upto a threshold maximum concentration for a given substance.

iv. Wavelength of light should be at the observation maxima of solution. This also gives the great sensitivity.